II. REQUEST FOR RECONSIDERATION UNDER 37 C.F.R. §1.111

A. Status of the Application

Rejections under 35 U.S.C. §102(b) and 35 U.S.C. §103(a) were withdrawn following Applicants Response to the Second Office Action mailed by Applicants on February 24, 2003. The current Action newly rejects the pending claims under 35 U.S.C. §112, first and second paragraphs.

B. Status of the Claims

Claims 1-74 were initially filed. Claims 33-74 have been withdrawn from consideration as directed to non-elected subject matter. Claim 22 has been amended herein to correct a typographical error. Therefore, claims 1-32 are currently pending in the application and presented herein for reconsideration.

C. Rejections Under 35 U.S.C. §112, First Paragraph

The Action newly rejects claims 1-32 under 35 U.S.C. §112, first paragraph, as not being enabled. In particular, the Action asserts that the specification has not provided enablement for: (A) use of the claimed method in the absence of a wash step, (B) use of the claimed method with molecules greater than 2000 Da, and (C) use of the claimed method where the labeled ligand comprises a nucleic acid. It is also stated that use of enzymes generally with the invention is not enabled. Applicants respectfully traverse as set forth below.

(1) A wash step is not necessary to the claimed method

Applicants note that a wash step is not required for use of the invention. The assumption that a wash step is necessary assumes that the concentration of labeled ligand will be sufficiently similar between the outside labeling solution and the inside of the periplasm of cells specifically binding the labeled ligand, and/or between cells with specific binding of the ligand and those without, that selection of cells with specifically bound labeled ligand will not occur. This is incorrect. The specific interaction of the binding protein and labeled ligand in the periplasm of the bacterium will ***retain*** and thus ***concentrate*** the labeled ligand inside the periplasm of ***only*** those cells with high affinity binding proteins. Such cells will become specifically labeled with the probe, while cells not expressing a binding protein having affinity for the labeled ligand will not. Thus, with or without a washing step, cells expressing ligand binding proteins become more labeled and can be identified on this basis alone.

If the foregoing were not correct, the method of the invention would not work regardless of the presence of washing. The function of the technique is based on differences in labeling between cells expressing a binding protein that is capable of selectively binding the labeled ligand and those that do not or have reduced affinities for the labeled ligand, thereby allowing selection of specifically labeled cells.

The ability to discern cells with strong binding of labeled ligand even from cells having moderate binding is illustrated in Example 2 of the working examples. There, Applicants describe studies investigating whether antibody mutants with increased binding affinity can be obtained by periplasmic expression/FACS screening, even when starting with an antibody that already exhibits very tight binding for the ligand. The results showed specific labeling to isolate a better mutant, even when starting with an antibody already exhibiting a sub-nanomolar K_D.

Although washing was used in this example, the fact remains that if cells with increased binding of labeled ligand were not different and distinguishable from those with little or no binding, selection would not have been achieved.

There is therefore no basis to conclude that a wash step is essential. Cells without specific binding are comparable to the labeling solution comprising non-specifically bound labeled ligand. While some non-specifically bound labeled ligand may be present, the cells in which specifically bound labeled ligand is present remain distinguishable. Restated, the kinetics of tight binding between ligand and binding protein insures higher labeling under near-equilibrium conditions in the bacterium. Bacteria that exhibit higher levels of labeling can then be separated based on the labeling, for example, by FACS as described in the specification. Still further, when FACS itself is employed this involves using a sheath fluid that moves the cells through the microfluidics of the instrument. This achieves the same thing as a wash step even assuming, *arguendo*, it was necessary.

(2) The invention is not limited to ligands of less than 2000 Da or specific classes of ligands

Applicants note that use of labeled ligands of more than 2000 Da is fully enabled by the specification. In this regard, it is initially noted that the references cited as teaching that molecules of only up to 900 Da can enter the cell or by specific transporter systems did not use conditions to permeabilize the gram negative bacteria. In contrast, the specification teaches hyperosmotic shock and other treatments that improve cell permeability and labeling significantly while maintaining cell viability. (See, e.g., Example 3). These treatments do not rely on any particular transporter system and thus are non-specific.

For instance, Example 8 of the specification (page 63) describes specific labeling of cells using an oligonucleotide (MW 2,384 Da) that contains digoxigenin on one end (the ligand) and fluorescein on the other end. Fig. 8 shows that an increase in FACS signal was obtained when cells expressing periplasmic scFv antibodies to digoxigenin were labeled with this probe using 5X PBS to permeabilize the outer membrane. The probe was greater than 2000 Da and thus the assertion of a lack of enablement is incorrect.

Applicants further note that additional studies have also demonstrated the enablement of the claims with even larger labeled ligands. Applicants direct the Examiner's attention in this regard to the paper by Chen *et al.*, (*Nature Biotechnology*, 2001, 19, 537-542; submitted as IDS ref. C54). There shown are studies demonstrating that oligonucleotide 10mers (4,897 Da) and even 20mers (8,727 Da) labeled with digoxigenin and a fluorescent label can successfully label *E. coli* in 5X PBS expressing scFv antibodies to digoxigenin in the periplasm. (see Fig. 3, page 539).

Further, studies carried out under the supervision of the inventors have also demonstrated that appreciable labeling was obtained using a peptide construct of molecular weight 2,505 Da or a polyethylene glycol conjugate of molecular weight 2,937 Da. In both cases the conjugates contained digoxigenin attached to one end and a fluorescent label on the other. Specific labeling was only seen with *E. coli* cells expressing the 26-10 antidigoxin antibody scFv in the periplasm. The results were achieved using polymyxin B nonapeptide (PMBN) as the outer membrane permeabilizer (specification, page 16). Should it advance the prosecution of the case, Applicants would be glad to provide a Declaration under 37 C.F.R. §1.132 in this regard.

What the foregoing studies and the specification demonstrate is that labeling of *E. coli* cells has been achieved using three entirely different classes of molecules (oligonucleotides,

oligopeptides and a polyethylene glycol derivative), with molecular weights between 2,000 Da and almost 9,000 Da, by adding reagents that partially disrupt the outer membrane. These permeabilization strategies, including 5X PBS and PMBN, act to ***destabilize the outer membrane*** as opposed to triggering any specific transport mechanism. The technique is therefore not specific to any class of molecule. The selectivity or lack thereof of any given cell membrane transport system is therefore irrelevant to the function of the claimed method. Knowledge of the exact identity of any transport systems involved with entry of larger ligands into the cell is also not required for use of the invention. As such, there is no basis to conclude that the invention is limited to any given class of labeled ligand.

(3) The use of nucleic acids has been enabled

Applicants note that the studies carried out by the inventors and working examples in the specification also demonstrate enablement for use of labeled ligands comprising nucleic acids. For example, the Chen *et al.* (2001) reference discussed above describes the use of labeled ligands comprising nucleic acids for detection in the periplasm of *E. coli* bacteria expressing a binding protein. In particular, oligonucleotide 10mers (4,897 Da) and even 20mers (8,727 Da) were labeled with digoxigenin and a fluorescent label, resulting in the successful labeling of *E. coli* in 5X PBS expressing scFv antibodies binding the digoxin in the periplasm. (see Fig. 3, page 539). The studies demonstrate enablement for use of nucleic acids.

Use of labeled ligands comprising nucleic acids is also described in Example 8 of the specification. This example demonstrates that a fluorescently-tagged oligonucleotide (2384 Da molecular weight) diffused into bacteria, and specific ligand-binding protein binding occurred. No significant DNA-labeled probe binding was observed in Example 8, and, because no evidence exists that this kind of interaction would present a significant problem for use of the

invention, the idea that DNA-labeled probe binding will prevent use of the invention must be regarded as speculation. The nucleic acids that are used as ligands can be easily distinguished from other nucleic acids because the ligand nucleic acids are tagged with a fluorescent (or other) labeling moiety.

The studies above demonstrate that nucleic acids can enter the periplasm. This further demonstrates that entry of labeled ligands into the periplasm is not limited to only certain types of molecules of very limited size. In this case, the nucleic acid is not used as a source of genetic information to be transcribed and translated into protein. Rather, the nucleic acid is being used as a scaffold to contain the ligand of the binding protein as well as a fluorescent label. Therefore, again, non-specific binding with native nucleic acids does not affect the ability to identify selectively bound labeled ligand.

(4) Use of enzymes is enabled

The Action also asserts that the specification does not enable use of enzymes generally. In particular, it is stated that, although use of *Fusarium solani* lipase cutinase is described in the specification, the specification has not shown that other enzymes and substrates diffuse across the bacterial outer membrane. It is stated that different substrates behave differently and require different transport systems to cross the outer membrane.

In response, it is again noted that the entry of molecules into the periplasm is not limited to only specific classes of molecules. The inventors describe membrane permeabilization methods that are non-specific. This is supported by the multiple different types of labeled ligands that were used and shown to successfully enter the periplasm, including nucleic acids, oligopeptides and a polyethylene glycol derivative, including substrate for *Fusarium solani*

lipase cutinase. There is no basis to conclude that only certain types of substrates will cross the outer membrane.

Further, no basis has been provided to conclude why the *Fusarium solani* lipase cutinase example is not representative of the claims in general. This constitutes a working example within the scope of the claims. Given the demonstrated ability to introduce a wide variety of molecules of different sizes into the *E. coli* periplasm, there is no basis to conclude that the example does not enable the full scope of the claims. Any suggestions to the contrary are unsupported speculation given the evidence presented by Applicants.

In view of the foregoing, Applicants respectfully request the removal of the rejection of claims 1-32 under 35 U.S.C. § 112.

D. Rejections Under 35 U.S.C. §112, Second Paragraph

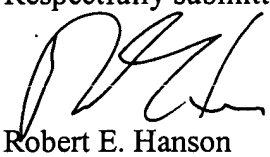
The Action rejects claims 1-32 under 35 U.S.C. §112, second paragraph as being incomplete for the omission of a wash step. Applicants respectfully traverse.

As described in detail above, a wash step is not required for the function of the invention and therefore this element need not be recited in the claims. Applicants need only recite those elements that are essential to the function of the invention. It is not required that non-essential embodiments from the disclosure be included in the claims. It is therefore respectfully submitted that the claims are fully definite and removal of the rejection is thus requested.

E. Conclusion

In light of the foregoing, applicants submit that all claims are in condition for allowance, and an early indication to that effect is earnestly solicited. The examiner is invited to contact the undersigned (512)536-3085 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



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